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Promotion of vesicular stomatitis virus fusion by the endosome-specific phospholipid bis(monoacylglycero)phosphate (BMP)

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ABSTRACT

Vesicular stomatitis virus (VSV) is a prototypic virus commonly used in studies of endocytosis and membrane trafficking. One proposed mechanism for VSV entry involves initial fusion with internal vesicles of multivesicular endosomes followed by back-fusion of these vesicles into the cytoplasm. One feature of endosomal internal vesicles is that they contain the lipid bis(monoacylglycero)phosphate (BMP). Here, we show that the presence of BMP significantly increases the rate of VSV G-mediated membrane fusion. The increased fusion was selective for VSV and was not evident for another enveloped virus, influenza virus. Our data provide a biological rationale for a two-step infection reaction during VSV entry, and suggest that BMP preferentially affects the ability of VSV G to mediate lipid mixing during membrane fusion.

1. Introduction

The endocytic pathway is responsible for the maintenance of the cell surface composition and is usurped by many viruses as a portal of entry into the cell [1,2]. Such viruses often take advantage of the low endosomal pH to trigger their uncoating and/or fusion as thus gain access to the cytosol for replication. While low pH is well recognized as a way to regulate the conformational changes occurring during virus entry, the role of other factors, such as lipid composition, has received comparably little attention. For alphavirus fusion, the strong dependence of membrane cholesterol and sphingolipids on virus entry is well documented [3–5] and cholesterol can also modulate influenza virus fusion pore formation [6], but the role of endosome-specific lipids for virus entry on lipids remains essentially unexplored.

Vesicular stomatitis virus (VSV) enters cells via clathrin-mediated endocytosis [7–9], with the elongated shape of the VSV particle necessitating a non-conventional process of partial clathrin coat assembly and local actin filament assembly for uptake into the cell [10]. Entry of the viral genome occurs by a low pH dependent process of membrane fusion mediated via the viral glycoprotein G [11]. VSV G shows unusual properties for a viral fusion protein, in that the conformational changes triggering membrane fusion are reversible [12]. Another unusual feature of VSV is that viral entry has been proposed to be mediated by a two-step infection process, with the initial fusion event occurring with internal vesicles of endosomal transport intermediates, followed by a "back-fusion" reaction of viral nucleocapsid-laden internal vesicles with the limiting membrane of late endosomes [13]. This mechanism has been disputed, and a more conventional fusion reaction directly with the limiting membrane of early endosomes proposed [8].

Membrane lipids play critical roles in the spatial organization of cells [14] and one feature of the internal vesicles of the endocytic pathway is that they contain a unique lipid, called either bis(monoacylglycero)phosphate (BMP) or lysobisphosphatidic acid (LBPA) [15,16]. BMP is thought to promote membrane fusion and membrane invagination in a low pH-dependent manner [17,18], and control the morphology and function of late endosomes and multivesicular bodies.

2. Materials and methods

2.1. Viruses and cells

Vesicular stomatitis virus (VSV) Indiana, strain Orsay (ATCC) was propagated in BHK cells (ATCC). Briefly, 80–90% confluent roller bottles of BHK cells were infected at a MOI of 0.01. After 24 h,

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the media was spun down and the supernatant was subjected to ultracentrifugation. The viral pellet was then applied to a sucrose step gradient to produce purified VSV. Viral protein concentration was determined using a Bradford protein assay.

Influenza A virus, strain X:31 (H3N2), was grown in 11 day old embryonated eggs and allantonic fluid was collected to prepare concentrated virus stock as described for VSV.

2.2. Liposomes

Palmitoyl-oleoyl-phosphocholine (POPC), palmitoyl-oleoyl-phosphoserine (POPS), palmitoyl-oleoyl-phosphatidic acid (POPA), bis(monoacylglycero)phosphate (BMP), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Labeled phospholipids *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) and Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rho-PE) were purchased from Invitrogen (Carlsbad, California). Large unilamellar vesicles (LUV) were prepared according to the extrusion method. Lipid films were obtained by subjecting chloroform dissolved lipid mixtures to high vacuum overnight. Lipid films were resuspended by addition of fusion buffer (20 mM MES, 30 mM Tris–HCl, pH 7) to 5 or 10 mM lipid concentration and incubated for 15 min at room temperature followed by vortexing for 15 min. Liposomes were then subjected to 10 freeze-thaw cycles, followed by 11 extrusions through 0.1 μm polycarbonate membrane using an Avanti mini extruder. Liposomes labeled with the FRET pairs Rho-PE and NBD-PE were made in the same manner with the addition of 0.6% each of NBD-PE and Rho-PE to the chloroform-dissolved lipid mixture.

2.3. Lipid mixing assay

Lipid mixing was determined using the method of Struck et al. [19]. Unlabeled and labeled liposomes were mixed at a 4:1 ratio to a total concentration of 110 μM lipid in fusion buffer at pH 7.0 with the temperature held at 37 °C and continuous stirring. Then VSV was added to a final concentration of 200 μg/ml total viral protein unless otherwise noted. To initiate fusion, hydrochloric acid was added to the solution until the desired pH was achieved. To end the reaction and obtain a measurement of 100% lipid mixing, reduced Triton X-100 was added to a final concentration of 0.2%. Changes in fluorescence were measured using a QM-6SE spectrofluorimeter (Photon Technology International, Birmingham, NJ) with excitation set at 467 nm and emission monitored at 530 and 581 nm. The extent of lipid mixing was determined using the formula:

$$F(\%) = \frac{f_t - f_0}{f_{100} - f_0} \times 100$$

where f_t is the fluorescence measurement at time t, f_0 is the initial fluorescence and f_{100} is the fluorescence after the addition of reduced Triton X-100. All measurements were taken in triplicate and averaged. The extent of lipid mixing 180 s after reaction initiation is shown (Table 1, Figs. 2–4).

Table 1
Extent of VSV G promoted lipid mixing over a range of pH values. The extent of lipid mixing promoted by VSV G, before reaction termination with reduced Triton-X100. The label −BMP refers to liposomes containing 4:5:1 POPC:POPS:cholesterol and the label +BMP refers to 2:2:5:1 POPC:BMP:POPS:cholesterol liposomes. Extent of lipid mixing is shown for several pH values corresponding to the rate information in Fig. 1. A starred (*) entry denotes lipid mixing below the limit of detection.

pH	−BMP		+BMP	
	Extent lipid mixing (%)	Standard deviation	Extent lipid mixing (%)	Standard deviation
5	27.3	1.6	45.1	7.3
5.25	22.1	3.2	34.2	1.0
5.5	18.0	0.6	30.4	1.6
5.75	11.0	0.8	23.8	1.6
6	5.1	0.2	14.6	1.2
6.25	1.3	0.3	6.3	0.1
6.5	0.6*	N/A*	2.1	0.5

3. Results and discussion

In order to determine whether BMP specifically promotes VSV G-mediated membrane fusion, we adapted a fluorescence resonance energy transfer (FRET)-based assay of lipid mixing [19] in order to mimic the fusion of VSV to the intralumenal vesicles of the multivesicular bodies. In this assay, purified VSV particles are mixed with labeled and unlabeled large unilamellar vesicles (LUVs). A hemi-fusion or fusion event between a VSV particle

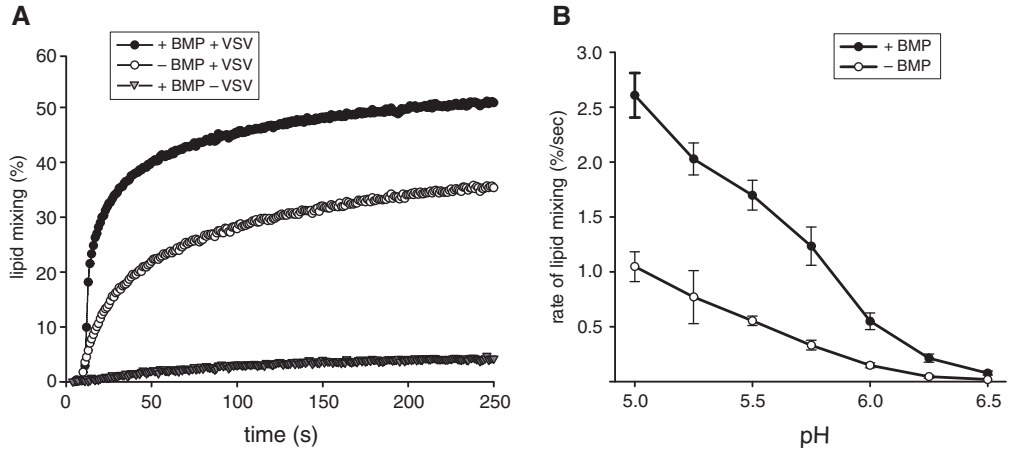
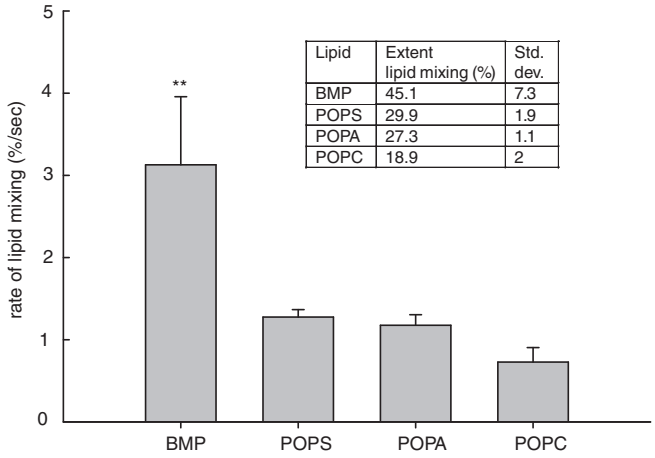
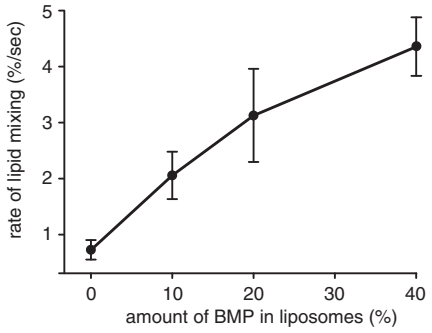


Fig. 1. The effect of BMP on VSV-G mediated lipid mixing. (A) Lipid mixing of VSV (200 μg/ml) with liposomes (110 μM) containing 4:5:1 POPC:POPS:cholesterol or 2:2:5:1 POPC:BMP:POPS:cholesterol liposomes in 20 mM MES, 30 mM Tris, pH 7 buffer was initiated by a decrease in pH to 5.0. Lipid mixing was followed by monitoring fluorescence intensity at 530 nm. Lipid mixing of 2:2:5:1 POPC:BMP:POPS:cholesterol liposomes were also monitored following a pH drop, in the absence of virus particles (−VSV). (B) The rate of lipid mixing during the first 10 s of the reaction was determined over a range of pH conditions with 4:5:1 POPC:POPS:cholesterol or 2:2:5:1 POPC:BMP:POPS:cholesterol liposomes. Each data point is averaged from three individual assays and error bars represent standard deviation of the mean. Data were analyzed using a Student's *t* test. *P* values are summarized as follows: ***P* value extremely significant ($P \leqslant 0.001$); **P* value very significant ($P = 0.001$–0.01); and *P* value significant ($P = 0.01$–0.05).



Lipid	Extent lipid mixing (%)	Std. dev.
BMP	45.1	7.3
POPS	29.9	1.9
POPA	27.3	1.1
POPC	18.9	2

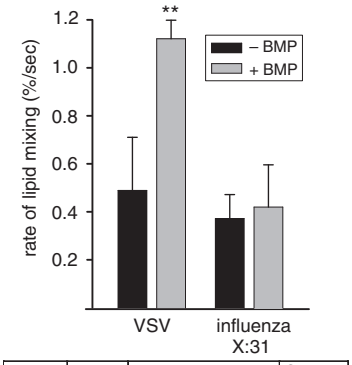
Fig. 2. Comparison of VSV promoted lipid mixing in the presence of anionic or zwitterionic lipids. A comparison of the rates of lipid mixing at pH 5 for VSV with 2:2:5:1 POPC:X:POPS:cholesterol liposomes, where X is the anionic or zwitterionic lipid indicated in the figure. Each bar is averaged from three individual assays and error bars represent standard deviation of the mean. Data were analyzed using a single factor ANOVA and the Tukey-Kramer method. A very significant P value ($P = 0.001–0.01$) is symbolized **. The accompanying table shows the extent of lipid mixing achieved before termination of the reaction.



BMP (%)	Extent lipid mixing (%)	Std. dev.
0	18.9	1.9
10	40.9	7.8
20	45.1	7.3
40	59.8	4.8

Fig. 3. The rate of lipid mixing by VSV G increases with increased BMP. The rate of lipid mixing, initiated by a drop in pH to pH 5, was examined with liposomes consisting of a range of BMP quantities (from 0% to 40% of the total liposome composition). The accompanying table shows the extent of lipid mixing achieved before termination of the reaction.

and a labeled liposome results in a dilution of the FRET pairs and an increase in NBD (donor) fluorescence, which is monitored by fluorescence spectroscopy. The extent of lipid mixing (L_t) was determined using the formula $L_t(\%) = [(I_t - I_0)/(I_{100} - I_0)] \times 100$ where I_t is the fluorescence measurement at time t, I_0 is the initial fluorescence and I_{100} is the fluorescence after the addition of reduced Triton X-100. We produced liposomes that contained BMP (2:2:5:1 BMP:POPC:POPS:cholesterol), and compared the extent of VSV-mediated fusion to liposomes without BMP (4:5:1 POPC:POPS:cholesterol). A representative experiment is shown in Fig. 1A. In the presence of VSV particles, both the initial rate of fusion and the extent of fusion were increased using liposomes that contained BMP. In the absence of virus particles, we observed only limited fusion of the liposomes with BMP (Fig. 1A). Initially, the lipid mixing reaction contains an excess of liposomes in the presence of limiting amount of VSV particles. During this stage, almost all of the increase in fluorescence signal is due to lipid mixing events involving liposomes and virus particles. In the later phase of the reaction, a complex mixture of species is present, and the fusion of those species contributes to the increase in fluorescence. Therefore, the initial rate of lipid mixing is the most accurate measure of the influence of the reaction conditions VSV G promoted lipid mixing. The rate of VSV-induced lipid mixing within the first 10 s of the fusion reaction for liposomes with and without BMP, at a range of pH values, is shown in Fig. 1B. At pH values below 6.5, there was a statistically significant difference in the rates of lipid mixing for liposomes with and without BMP. Further, lipid mixing experiments with liposomes of other compositions show that the extent and initial rate of lipid mixing measured using 2:5:1:2 POPC:POPS:BMP:cholesterol liposomes is not achieved in liposomes containing BMP but not POPS.



Virus	BMP	Extent lipid mixing (%)	Std. dev.
VSV	-	10.9	2.8
	+	18.7	2
X-31	-	18.2	3.1
	+	14.3	3.8

Fig. 4. Virus specificity of lipid mixing rate acceleration by BMP. A comparison of the rate of lipid mixing for VSV and influenza virus, each at a concentration of 100 μg/ml, with 4:5:1 POPC:POPS:cholesterol or 2:2:5:1 POPC:BMP:POPS:cholesterol liposomes at pH 5. Each bar is averaged from three individual assays and error bars represent standard deviation of the mean. Data were analyzed using a Student's t test. A very significant P value ($P = 0.001–0.01$) is symbolized **. The accompanying table shows the extent of lipid mixing achieved before termination of the reaction.

It has long been known that the VSV G protein can specifically interact with lipids, and a selective interaction with phosphatidylserine was originally proposed to account for virus-receptor interactions during entry into the cell [20], although this has recently been disputed [21]. In general, VSV G seems to have an affinity for negatively charged phospholipids [22,23]. BMP is also anionic, and so its charged head group could account for the enhancement of VSV G-mediated membrane fusion. In order to assess the effect of negative charge, we added the anionic phospholipids POPS, POPA, and the zwitterionic lipid POPC to the liposomes and compared the rate of lipid mixing promoted by VSV to the rate with BMP containing liposomes (Fig. 2). The lipid mixing assay showed that there was a statistically significant difference in the initial rate of lipid mixing when the liposomes contained BMP compared to liposomes that contained POPS, POPA and POPC. Therefore, negative charge does not appear to be the sole way of enhancing VSV G-mediated membrane fusion, as other negatively charged lipids showed only minimal enhancement of fusion, as shown in Fig. 3. Another possible explanation for the effects of BMP is that the increased fusion is due to increased binding between VSV and BMP-containing liposomes. However, VSV-liposome flotation assays show that, under the experimental conditions used here where liposomes are in vast excess over viral particles, there is no significant difference in the amount of VSV bound in the presence or absence of BMP (data not shown). In fact, Western blot shows that greater

than 95% of the viral particles are bound to liposomes in the absence and presence of BMP.

The membranes of late endosomes have been reported to contain approximately 20% BMP, which provided us a biologically relevant liposome composition to use in our initial experiments. In order to confirm the effect of BMP on VSV G-mediated lipid mixing, we varied the amount of BMP present in the liposomes and examined the rate of lipid mixing at pH 5. As shown in Fig. 3, the rate of lipid mixing increases with increasing amounts of BMP present in the liposome composition.

To date, a two-step infection process involving two discrete fusion reactions has only been proposed during entry of VSV. We therefore examined whether BMP might promote membrane fusion in other viral systems. We tested the effect of BMP on membrane fusion mediated by the hemagglutinin (HA) of influenza virus. As shown in Fig. 4, our FRET-based assay clearly showed that when liposomes were fused with influenza virus (strain X:31), the addition of BMP had no significant effect on the rate of fusion. In contrast, BMP had a profound effect on the rate of lipid mixing promoted by VSV G, suggesting that an interaction with BMP is specific property of VSV.

Our data show that the endosome-specific lipid BMP promotes membrane fusion mediated by the VSV G protein, and provide a possible rationale for two-step endosomal infection during VSV entry. Gruenberg and colleagues considered that BMP was involved in the back-fusion of VSV G-containing endosomal vesicles via the action of Alix [13], rather than having a direct role in virus-induced fusion. Our data suggest that BMP can also directly promote the fusion of the VSV particle, at least to the lipid mixing stage. It has previously been reported that the presence of BMP in liposomes allows spontaneous fusion in a pH-dependent manner [17]. While some degree of BMP-induced liposome fusion in the absence of virus particles was observed in our experiments, this was limited compared to fusion promoted by VSV G either in the presence or absence of BMP.

Our studies reveal an important role for BMP in directly modulating VSV G-mediated membrane fusion. While the presence of negatively charged phospholipids (e.g. cardiolipin) is thought to non-specifically to induce membrane fusion [24], our data showing a specific effect on VSV, combined with a pH-dependence of BMP-stimulated fusion, argue that the in vitro data presented here are of biological importance. Indeed, a notable feature of our experiments is that whereas BMP promoted VSV G-induced membrane fusion, it did not significantly affect influenza HA-mediated fusion. The fusion peptides of G and HA are very different; whereas influenza HA has an external α-helical fusion peptide that inserts into the lipid bilayer at an oblique angle [25,26], VSV G has an internal fusion peptide composed of a bipartite loop structure that is thought to dip into the bilayer [27,28].

The mechanism by which BMP induces its differential effect on VSV is presently unclear, but it is likely to be due to an interaction with the viral fusion peptide. BMP is a unique derivative of phosphatidylglycerol that shows unusual features for a membrane phospholipid [29]. One possibility for the effect of BMP is related to its shape; however whether the lipid is a cone, inverted cone or cylindrical in shape is unclear [29]. It has previously been reported that influenza virus HA-mediated fusion was inhibited by the inverted cone-shaped lipid lysophosphatidic acid and promoted by the cone-shaped oleic acid [30]. As BMP showed no effect on influenza virus fusion (Fig. 4), it seems unlikely that an equivalent mechanism would underlie the effect of LPBA on VSV G-although such a conclusion would need more evidence for the physical behavior of BMP in lipid bilayers.

The role of BMP and the two-step model of VSV entry remain controversial. It may be that depending on the composition of endosomal domains in different cell types, the virus can fuse either directly from the early endosome, or enter via back-fusion in late endosomes. Cell types with higher amounts of endosomal BMP may re-direct fusion toward the lumen of the MVB, rather than towards the cytosol. Such a variation in the site of fusion during virus entry might be facilitated by the relatively broad range of pH values (from approximately 6.2–5.0) that have been shown to activate the VSV G fusion machine [28,31].

In conclusion, we show here a specific promotion of VSV G-mediated membrane fusion by BMP. A similar role for BMP has also been reported for dengue virus [32], where it is proposed that interaction with anionic phospholipids such as BMP regulate fusion within the endosomal network and protect the virus against premature irreversible restructuring. It will be interesting to determine the physiological role of BMP in live cells, for VSV as well as other pathogens that navigate endosomal compartments during their entry into host cells [33].

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